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3 PART III APPLICATION IN EPIDEMIOLOGY

3.1 CYP2D6 AND CYP2C19 ACTIVITY IN A LARGE POPULATION OF DUTCH HEALTHY VOLUNTEERS: INDICATIONS FOR ORAL CONTRACEPTIVE-RELATED GENDER DIFFERENCES[#]

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3.1.1 Abstract

Objective: We examined a large database containing results on CYP2D6 and CYP2C19 activity of 4301 Dutch volunteers phenotyped in the context of various clinical pharmacology studies.

Methods: The subjects were given 22 mg dextromethorphan, 100 mg mephenytoin and 200 mg caffeine. For CYP2D6, the dextromethorphan / dextrophan metabolic ratios in urine samples taken for a subsequent 8 hours were used. Dextromethorphan and dextrophan were quantified by reversed-phase high performance liquid chromatography. For CYP2C19 similarly obtained (R)-mephenytoin and (S)-mephenytoin ratios were used. (S)-mephenytoin and (R)-mephenytoin were analysed and quantified by enantioselective capillary gas chromatography. In addition, CYP2C19 PM subjects were reanalysed after acidic pre-treatment of urine samples to confirm the PM status.

Results: The investigated population mainly comprised Caucasian (98.9%) males (68%). The age ranged from 18 to 82 years. For CYP2D6 it was found that 8.0% of the subjects was poor metabolizer (PM). The average metabolic ratio was 0.014 ± 0.033 for subjects who showed extensive metabolizing activity (EM) and 5.4 ± 7.6 for PM subjects. For CYP2C19 it was found that 1.8% of the subjects was poor metabolizer. The metabolic ratio was 0.162 ± 0.124 for EM subjects and 1.076 ± 0.040 for PM subjects. Within the EM group the metabolic ratio in females was significantly lower for CYP2D6 (-20%) and significantly higher for CYP2C19 (+40%) compared to males. For PMs there was no such difference for CYP2D6 ($p = 0.79$) or CYP2C19 ($p = 0.20$). Oral contraceptive use significantly decreased the CYP2C19 activity with 68% for mephenytoin as compared to non-OC using females.

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Conclusions: For CYP2D6, the PM incidence (8.0%) is in accordance with literature data. The CYP2C19 PM incidence (1.8%) is low compared to reports from other European countries. For mephenytoin, the acidification procedure has shown very important for the confirmation of CYP2C19 PMs. In EM females compared to EM males CYP2D6 activity is increased and CYP2C19 activity is reduced. Particularly for CYP2C19 this reduction is substantial and the most pronounced in the age range from 18 to 40 years. For CYP2C19 the reduced activity is associated with the use of oral contraceptives.

Key words CYP2D6, Dextromethorphan, CYP2C19, Mephenytoin, Dutch Population, Gender Differences

3.1.2 Introduction

CYP isoenzymes are known to be important catalysts for oxidative biotransformation of both endogenous and exogenous compounds [1]. Drugs are often metabolised by a limited number of iso-enzymes of Cytochrome P450 (CYP). Due to genetic polymorphism of some of these iso-enzymes, standard medication may lead to a large inter-individual variation in drug levels causing a large variability in therapeutic response and enhancement of adverse drug effects. Although the recognition of extensive (EMs) and poor metabolizers (PMs) with respect to many cardiovascular and psychotropic agents occurred already 15 years ago, phenotyping and/or genotyping for these iso-enzymes is rare in current clinical practice [2].

CYP2D6, debrisoquine 4-hydroxylase, a well-studied isoform of CYP P450, is involved in the metabolism of many widely prescribed drugs, including beta-adrenergic receptor antagonists, antiarrhythmics, antidepressants and neuroleptics [3]. The individual status of CYP2D6 activity can be probed with several drugs such as debrisoquine, sparteine, metoprolol and dextromethorphan. Poor activity of the enzyme (PM phenotype) is an autosomal recessive trait that affects approximately 7% of Caucasians and 1% of Orientals. The opposite phenomenon called ultrarapid metabolism also exists and is caused by gene amplification [4]. With standard medication this phenotype will suffer from subtherapeutic plasma concentrations and thus from decreased drug response.

The polymorphic enzyme CYP2C19 hydroxylates (S)-mephenytoin on the 4' position. Here, too, there are marked interracial differences with respect to Caucasians and Orientals: the PM incidence is approximately 3% in Caucasian and 13 to 23% in Orientals [3]. Two null-alleles in the CYP2C19 gene have been described to be responsible for approximately 83% of all PMs in Caucasians and 100% of all PMs in Orientals [5, 6]. Recently, gender differences in (S)-mephenytoin 4'-hydroxylase have been found in a Chinese population[7]. It was concluded that higher CYP2C19 activity in females exists in both phenotyped EMs and genotyped homozygous EMs compared with the same group of males.

In the present study, we examined a large database containing phenotyping results on CYP2D6 (dextromethorphan) and CYP2C19 (mephenytoin) activity of 4301 Dutch healthy volunteers phenotyped in the context of clinical pharmacology studies.

3.1.3 Materials and Methods

Subjects

Four thousand three hundred and one (4301) unrelated subjects (3299 males and 1002 females) were evaluated. Sixteen hundred and sixty-three (1663) subjects (1469 males and 194 females) were phenotyped for CYP2D6, whereas 2638 subjects (1830 males and 808 females) were phenotyped for both CYP2D6 and CYP2C19 expression. The latter group was also phenotyped for N-acetyltransferase using caffeine as substrate; these data will not be discussed in this paper, however. All subjects were healthy volunteers and were phenotyped in the context of various clinical pharmacology studies. Most of them were recruited from the northern part of The Netherlands.

Health assessment was made by medical history, which was without major pathology, and by an eligibility screening including clinical chemistry, haematology and urinalysis before drug administration. None of the volunteers had a history of alcohol abuse, drug addiction or a smoking habit of more than 15 cigarettes daily. The participants were not allowed to take any medication (with the exception of oral contraceptives or paracetamol) 14 days before phenotyping. Subjects gave their written informed consent and the phenotyping protocol was approved by an independent Medical Ethics Committee: 'Stichting Beoordeling Ethiek Bio-Medisch Onderzoek', Assen, The Netherlands.

Phenotyping procedure

The subjects emptied their bladders before they took their medication: 10 mL of dextromethorphan hydrobromide monohydrate syrup containing 2.2 mg.mL⁻¹ dextromethorphan (Samenwerkende Apothekers B.V., Utrecht, The Netherlands), 200 mg caffeine powder (Genfarma, Maarssen, The Netherlands) and tablet containing 100 mg racemic mephenytoin (Epilanex[®]; Gerot Pharmazeutika, Vienna, Austria). Total urine was collected for 8 hours after intake of the medication and stored at -20 °C until analysis.

Bioanalysis

Dextromethorphan and dextrophan were analysed by reversed phase high performance liquid chromatography. Sample preparation was performed by liquid-liquid extraction with a mixture of n-hexane and n-butanol (90:10,v/v) after hydrolysis with 12 M hydrochloric acid (100 °C for 90 minutes) to release conjugated dextrophan. Separation was performed on a C₈ analytical column and a mobile phase containing phosphate buffer (pH = 4), triethylamine and acetonitrile. Quantitation was performed by fluorescence detection (excitation: 230 nm; emission: 310 nm). The assay method was validated on selectivity, sensitivity, recovery, accuracy, precision, linearity and stability. During analysis, urine samples spiked with dextromethorphan and dextrophan at three concentration levels were used for in-process quality control. Urinary

dextromethorphan/dextrorphan ratio was used as an indication for metabolic activity. According to Schmid et al. subjects with metabolic ratios ≥ 0.3 were classified as poor metabolizer (PM) and ratios < 0.3 were classified as extensive metabolizers (EM)[8]. The validated range of the assay method was 0.10 to 7.00 mg.L⁻¹ for dextromethorphan and 2.5 to 60.0 mg.L⁻¹ for dextrorphan. Within-run coefficients of variation (CVs) were below 8.0% for both compounds and between-run CVs were 17.6% for both compounds at all concentration levels. Dextromethorphan and dextrorphan in urine showed no notable deterioration during storage at -20 °C for at least six months, nor after repeated freezing and thawing (n = 5). None of the urine samples was stored for more than six months before analysis.

(S)-mephenytoin and (R)-mephenytoin were analysed by an enantioselective capillary gas chromatography assay based on the method described by Wedlund et al. [9]. Sample preparation was performed by liquid-liquid extraction with diethylether. Separation was performed by a Chirasil-Val capillary column (Alltech Nederland B.V., Breda, The Netherlands) with helium as carrier gas. Quantitation was performed using a nitrogen-phosphorous selective detector. The assay method was validated as described above. In-process quality control was performed by analysing urine samples spiked with racemic mephenytoin at four concentration levels. The urinary (R)-mephenytoin and (S)-mephenytoin excretion ratio was used as metabolic ratio. Subjects with S/R-ratios ≥ 0.8 were classified as poor metabolizer whilst subjects with ratios < 0.8 were classified as extensive metabolizer [10]. In addition, all CYP2C19 PMs, except for three subjects, were reanalysed after acidification of the urine before extraction with HCl (12M) to confirm the PM status[11].

The validated range of the assay method was 10.0 to 500 µg.L⁻¹ for both enantiomers. Within-run CVs were below 3.6% and between-run CVs were below 11.4% at all concentration levels. The S/R-ratio in spiked urine samples stored at -20°C was stable over a period of approximately two years and was also stable after repeated freezing and thawing (n = 5). None of the urine samples was stored for more than six months before analysis.

Statistical analysis

All data was collected in Microsoft Excel (Microsoft, Redmond, USA) in which all database handlings and statistical analyses (descriptive statistics and t-tests) were performed. SAS version 6.12 (SAS Institute Inc., Cary, USA) was used for the Chi-square test.

3.1.4 Results

Demographics

The population investigated mainly comprised Caucasian (98.9%) male (68%) volunteers. Of the total population 0.5% was Negroid and 0.6% was of Oriental origin. The age ranged from 18 to 81 years (26 ± 11) for males and 18 to 82 years (34 ± 18) for females. The higher mean age of women is due to a large number of postmenopausal women who were recruited for several clinical studies.

CYP2D6 Phenotyping Results

Table 1 summarizes the phenotyping data. For CYP2D6 8.0% of the volunteers was a poor metabolizer. Based on the Chi-square distribution (95%-confidence) the incidence of PMs varied between 7.2% and 8.8%. The PM incidence in females (7.2%) was lower than in males (8.2%) but this was not statistically significant using the Chi-square test ($p = 0.31$). The PM incidence in non-Caucasians (2%) appeared to be different from the Caucasian population (8.0%) but clear conclusions cannot be drawn because of the low number of non-Caucasians in our population.

Table 1: CYP2D6 Phenotyping results in a Dutch population, NA: not applicable

	Number of volunteers			PMs (%)	Ratio Total		Ratio EM		Ratio PM	
	Total	EMs	PMs		Mean	SD	Mean	SD	Mean	SD
Total	4301	3959	342	8.0%	0.440	2.584	0.014	0.033	5.377	7.593
Male	3299	3029	270	8.2%	0.458	2.544	0.014	0.034	5.433	7.227
Female	1002	930	72	7.2%	0.382	2.716	0.012	0.029	5.169	8.884
Caucasian	4252	3911	341	8.0%	0.445	2.599	0.014	0.033	5.390	7.600
Others	49	48	1	2.0%	0.036	0.146	0.016	0.024	1.022	NA

The average metabolic ratio was 0.014 ± 0.033 for EM subjects and 5.4 ± 7.6 for PM subjects. Figure 1 shows the frequency distribution of the metabolic ratios for CYP2D6. As expected two subpopulations can be seen on either side of the antimode. Taking an error of the ratio, due to analytical errors, of 20% (antimode range: -1.43 to -1.02) into account, it can be calculated that 0.6% of the ratios may be misclassified. In 47% of all subjects (all EMs), the dextromethorphan concentration was below the limit of detection (0.05 mg.L^{-1}). For metabolic ratio calculations of these samples, the dextromethorphan concentration was set at 0.05 mg.L^{-1} for calculation purposes. In 1.2% of all subjects (all PMs) the dextromethorphan concentration was below the limit of detection (0.10 mg.L^{-1}) for which the dextromethorphan concentrations were set at 0.10 mg.L^{-1} for calculation purposes. For dextromethorphan, large inter-individual differences were found in the urinary ratios for both EMs and PMs (Figure 1). For extensive metabolizers the concentration for dextromethorphan was $0.164 \pm 0.366 \text{ mg.L}^{-1}$ ($n = 3960$) and for dextromethorphan it was $17.9 \pm 12.1 \text{ mg.L}^{-1}$. For poor metabolizers the concentration for dextromethorphan was $2.43 \pm 1.59 \text{ mg.L}^{-1}$ ($n = 342$) and for dextromethorphan it was $0.92 \pm 4.35 \text{ mg.L}^{-1}$.

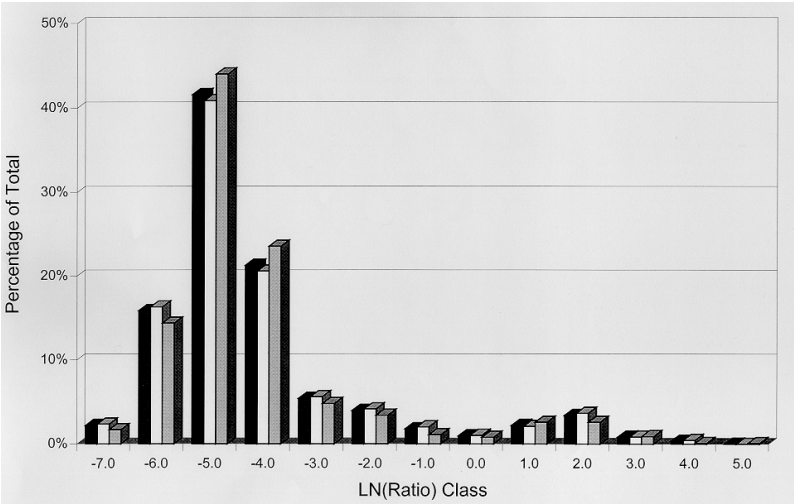


Figure 1: Histogram of the urinary dextromethorphan/dextrorphan ratio distribution as a percentage of total and differentiated to gender.

The number of phenotyped persons on CYP2D6 is 4301: 3299 males and 1002 females. An antimode of 0.3 ($\ln(\text{ratio}) = -1.2$) was used to distinguish extensive from poor metabolizers. ■: Total; □: Men; ▒: Women

CYP2C19 Phenotyping Results

Table 2 summarizes the CYP2C19 phenotyping data. For CYP2C19 1.8% of the subjects was found a poor metabolizer.

Table 2: CYP2C19 Phenotyping results in a Dutch population, NA: not applicable; NC: not characterized; samples initially identified as PM, but after acidification procedure characterized as EM (Increase of S/R ratio above 1.4)

	Number of volunteers				PMS	S/R Ratio Total		S/R Ratio EM		S/R Ratio PM	
	Total	EMs	PMs	NC	(%)	Mean	SD	Mean	SD	Mean	SD
Total	2638	2584	48	6	1.8%	0.178	0.174	0.162	0.124	1.076	0.040
Male	1830	1787	39	4	2.1%	0.160	0.171	0.140	0.105	1.080	0.040
Female	808	797	9	2	1.1%	0.219	0.172	0.209	0.147	1.061	0.033
Caucasian	2613	2560	47	6	1.8%	0.178	0.173	0.161	0.124	1.077	0.040
Others	25	24	1	0	4.0%	0.223	0.222	0.189	0.143	1.049	NA

Based on the Chi-square distribution (95%-confidence) the incidence of PMs varies between 1.3% and 2.4%. The PM incidence in females (1.1%) is lower than in males (2.1%) but this is not statistically significant (Chi-square test $p = 0.072$). The PM incidence in non-Caucasians (4.0%) appeared to be different from the Caucasian population (1.8%). The number of samples initially identified as PM but after acidification characterized as EM was 0.23% of all

subjects and 11.1% of the initially identified PM samples. The metabolic ratio was 0.162 ± 0.124 for EM subjects and 1.076 ± 0.040 for PM subjects.

Figure 2 shows the frequency distribution of S/R-ratios for CYP2C19. Two subpopulations can be seen on either side of the antimode. Taking an error of the ratio, due to analytical errors, of 10% (antimode range: 0.72 to 0.88) into account, it can be calculated that 0.3% may be misclassified. It can be seen that there is a difference between the distribution of male and female subjects. In general, EM females have a higher S/R-ratio compared to EM males. For calculation purposes, the S/R-ratio was set at 0.02 in case the sample had a non-measurable (S)-mephenytoin peak height. In 6.8% of all subjects, the (S)-mephenytoin peak height was equal to or below the limit of detection.

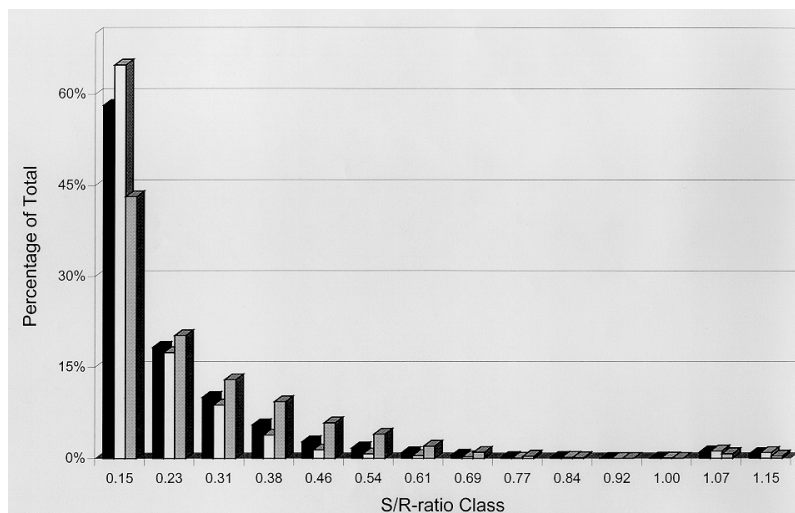


Figure 2: Histogram of the urinary mephenytoin S/R-ratio distribution as a percentage of total and differentiated to gender.

The number of phenotyped persons on CYP2C19 is 2638: 1830 males and 808 females. An antimode of 0.8 was used to distinguish extensive from poor metabolizers. The PM status was confirmed by the acidification method. ■:Total; □: Men; ▒: Women

Gender Differences

In Figures 3 and 4, a comparison between the genders for dextromethorphan and mephenytoin metabolism is given. From the data of EM samples it can be concluded by application of the students t-test that there is a significant gender difference in the metabolic ratio for both CYP2D6 ($p = 0.035$) and CYP2C19 ($p < 0.01$). The p-value of the t-test on CYP2C19 is only indicative due to the highly skewed distribution of the ratios. The metabolic ratios in females are 20% lower for CYP2D6 and 40% higher for CYP2C19. This indicates a significantly increased activity for CYP2D6 and a reduced activity for CYP2C19 in females compared to males.

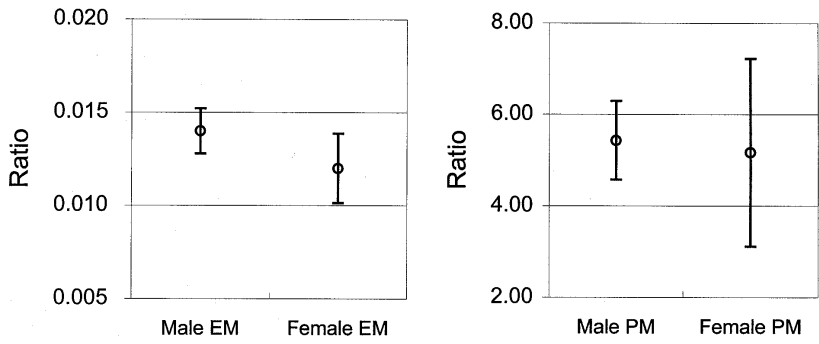


Figure 3: Schematic presentation of the mean metabolic ratios and 95%- confidence limit around the mean for CYP2D6 enzyme activity.

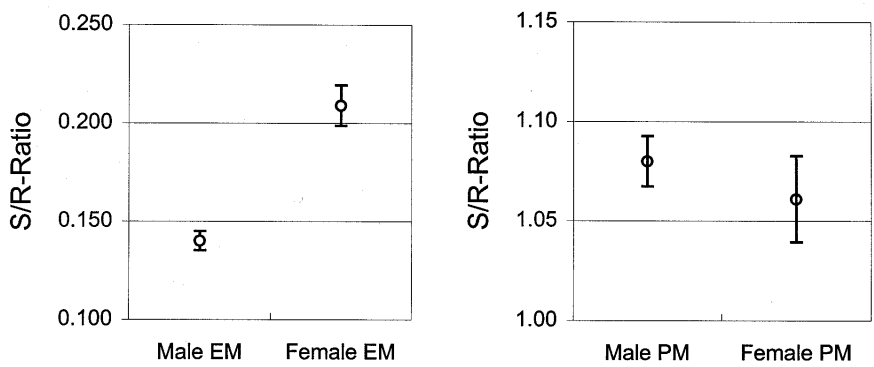


Figure 4: Schematic presentation of the mean metabolic ratios and 95%- confidence limit around the mean for CYP2C19 enzyme activity

For PMs there is no such difference for CYP2D6 ($p = 0.79$) neither for CYP2C19 ($p = 0.20$). From these data, we concluded that there is no significant gender difference between the PM incidence for both CYP2D6 and CYP2C19. To check whether the differences in metabolic ratio for EMs were caused by age differences between men and women in our database, an alternative database was constructed from the original one in which no age differences were present. Therefore, the original database of all EMs was split into eight classes (each ten years of age) and in each class equal numbers of men and women were randomly selected. The results are summarized in Table 3. It can be concluded that the differences between men and women are not caused by the different ages between men and women in our database.

Table 3: Results of a t-test between men and women EMs in a database constructed with equal mean ages for men and women (randomly selected subjects)

	<i>Dextromethorphan: CYP2D6</i>		<i>Mephenytoin: CYP2C19</i>	
	<i>Men</i>	<i>Women</i>	<i>Men</i>	<i>Women</i>
Mean age (years)	31.4	31.2	31.7	31.3
Mean metabolic ratio	0.0154	0.01195	0.144	0.218
Number of observations	833	833	720	720
P value (two tail)	0.037		< 0.001	

Influence of Age and Oral Contraceptives

To investigate the relationship between metabolic ratio and age the mean ratios per year of age and second order polynomial trendlines were plotted (Figures 5 and 6). Due to the small sample size, the data above 70 years are summarized in one category.

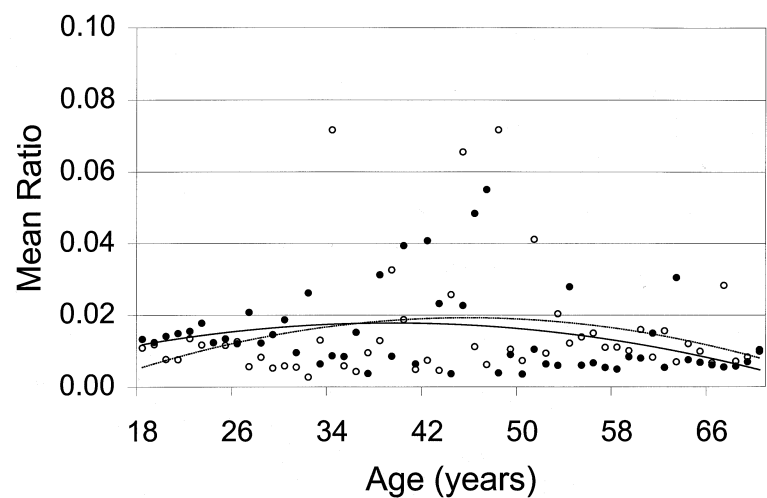


Figure 5: Mean dextromethorphan metabolic ratio (CYP2D6) per year of age and per gender plotted versus age. O: female; x: male; ----: trend line female; — : trend line male

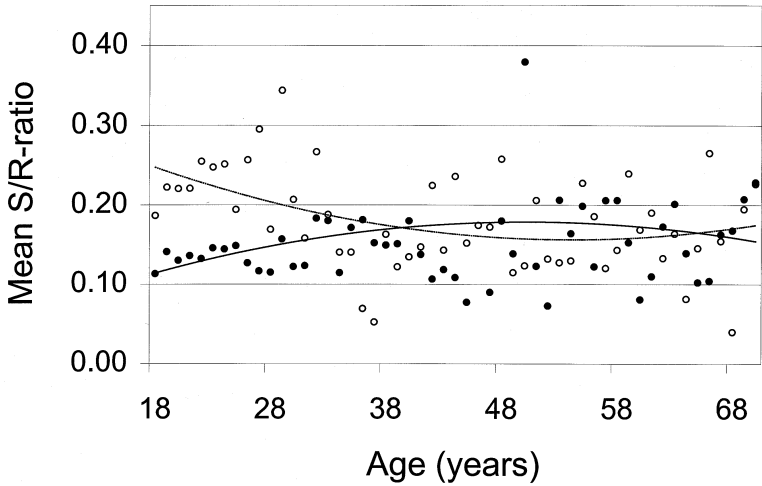


Figure 6: Mean mephenytoin S/R-ratio (CYP2C19) per year of age and per gender plotted versus age. O: female; x: male; ----: trend line female; — : trend line male

There is no clear relation between age and CYP2D6 enzyme activity for EMs, although the mean metabolic ratios are slightly increased in middle-aged categories (Figure 5). For CYP2C19, the data suggest that there is an age dependency for both men and women (Figure 6).

For EM males the CYP2C19 enzyme activity is high at eighteen years of age, it gradually decreases to approximately 40 years of age where it levels off at a S/R-ratio of approximately 0.15. For EM females, an opposite trend is seen: a low enzyme activity at 18 years of age and an increase to reach a plateau at approximately 40 years of age.

In Table 4 and 5, the results of males and females divided into two age categories: up to 40 years and above 40 years are shown.

Table 4: Results of a t-test between men and women EMs for CYP2D6 in a database differentiated according to age

	<i>Dextromethorphan: CYP2D6</i>			
	<i>Men</i>	<i>Women</i>	<i>Men</i>	<i>Women</i>
Age (years)	<40	<40	> 40	> 40
Mean metabolic ratio	0.0146	0.0109	0.0133	0.0141
Number of observations	2782	638	247	292
P value (two tail)	0.012*	0.117**	0.560**	0.737*

*: P-value: differences between men and women

**: P-value: differences between <40 and >40 of the same gender

From Table 4 it can be seen that there is significant difference between genders below 40 years ($p = 0.012$). There is no significant difference within gender below and above 40 years (male $p = 0.560$, female $p = 0.117$). From Table 5, it can be concluded that metabolic ratios below 40 years of age differ significantly between genders, but that there is no significant difference above 40 years. It can also be seen that there is a significant difference for both males and females below and above 40 years.

Table 5: Results of a t-test between men and women EMs for CYP2C19 in a database differentiated according to age

	<i>Mephenytoin: CYP2C19</i>			
	<i>Men</i>	<i>Women</i>	<i>Men</i>	<i>Women</i>
Age (years)	<40	<40	> 40	> 40
Mean metabolic ratio	0.136	0.227	0.171	0.172
Number of observations	1587	541	200	256
P value (two tail)	< 0.001*	< 0.001**	< 0.001**	0.95*

*: P-value: differences between men and women

**: P-value: differences between <40 and >40 of the same gender

Since females below 40 years of age are likely users of oral contraceptives (OCs) and above 40 years the OC use is less likely, the influence of OCs on enzyme activity was investigated. In the total volunteer population of Pharma Bio-Research the incidence of OC use is approximately 53% of all females. For comparison a subpopulation of females ($N = 115$) was selected from all phenotyped females ($N = 1002$) of which there was evidence on OC use at the moment of phenotyping. The results of analysing this subset are summarized in table 6. In the subset the incidence of OC use was lower as compared to our total volunteer population (38 %) which may be due to a relatively large number of postmenopausal women in the subset.

It can be seen (Table 6) that for EM females there is a significant difference between OC users and non-users in CYP2C19 enzyme activity.

Table 6: Results of a t-test between female OC users and non-users in 115 females selected from the database

	<u>CYP2D6 ACTIVITY</u>				<u>CYP2C19 ACTIVITY</u>			
	<u>OC USER</u>		<u>NON USER</u>		<u>OC USER</u>		<u>NON USER</u>	
	Mean	N	Mean	N	Mean	N	Mean	N
Ratio (all)	0.005	43	0.012	66	0.294	44	0.175	69
Ratio (< 40 Y)	0.005	43	0.013	36	0.294	44	0.177	37
Ratio (> 40 Y)	NA	0	0.010	30	NA	0	0.173	32
P value (two tail)	0.08*		0.70**		<0.002*		0.89**	

NA not applicable; * t-test between OC users and non users; ** t-test between non-OC users below and above 40 years of age

The mean S/R-ratio increased from 0.175 (non-users) to 0.294 (OC users), showing a decreased enzyme activity of 68%. For CYP2D6 no significant difference is observed ($p = 0.08$) although caution should be taken due to the skewed distribution.

Furthermore, it can be seen that in this subset no significant age difference was observed for both CYP2D6 ($p = 0.70$) and CYP2C19 ($p = 0.89$).

For CYP2C19 a comparison between males (Table 2) and non-OC using females (Table 6) indicates a significantly increased S/R-ratio (0.140 vs 0.175; $p = 0.008$) in females indicating a reduced CYP2C19 activity.

3.1.5 Discussion

For CYP2D6, the incidence of PMs (8.0%) that we report here is in accordance with literature data on the incidence in Caucasian populations [12]. It also agrees with a study in which 7.5% of 172 healthy Dutch subjects were found to be PM for CYP2D6 [13]. The incidence of PMs for CYP2C19 (1.8%) was also in accordance with literature data in Caucasian (2-6%) and a Dutch population (2.3%) [14, 15]. The CYP2C19 PM incidence in other European countries varies between 6.0 % (France; $n = 132$) and 2.5% (Denmark; $n = 358$) [16, 17]. Apparently the CYP2C19 PM incidence in The Netherlands is low compared to other European countries.

Recently it was suggested that volunteer databases of drug study units do not reflect the general composition of the population due to selective loss of PMs [18]. When volunteers experience adverse events this might make participation in a next trial less likely and therefore those who are more likely to experience adverse events (PMs) will drop out of the database. In our study however, this is not very likely since the majority of volunteers of our database was phenotyped just before or after their first clinical study in our institute. Therefore, we are confident that our population is representative for the population in general.

For dextromethorphan, large inter-individual differences were found in the urinary ratios for both EMs and PMs. This is in accordance with the findings of others and comparable to the results of a large study in Germany [19]. This variation may be caused by interindividual variations in other metabolizing enzymes such as CYP3A-isoforms [20], non-genetic factors [12] or different levels of expression of CYP2D6 in EMs.

For both dextromethorphan and mephenytoin the analytical methods are adequate for phenotyping, as indicated by the low assumed incidences of possible misclassification, 0.6% and 0.3% respectively. For mephenytoin, the acidification procedure appeared to be very important for the confirmation of CYP2C19 PMs. Omitting this procedure would have caused false positive PM classification of 11.1% of all PMs.

An interesting observation is the marked gender difference for mephenytoin which can be seen, although less pronounced also for dextromethorphan (Figures 3 and 4). For CYP2C19, the data strongly suggest a difference within gender but this appears to be related to age and the use of OCs. The concept of gender differences as a factor important in drug pharmacokinetics and

pharmacodynamics is not new [21]. In the early seventies, it was already reported that the metabolism of antipyrine is influenced by gender [22]. For CYP3A4, women appear to have higher enzyme activity compared to men [21]. For CYP2D6, only few data are available yet, but no gender-related difference is apparent [21]. For CYP2C19, it was reported that excretion of 4'-hydroxymephenytoin was higher in males than in females [13]. These results were confirmed by several studies reviewed by Harris et al., who concluded that CYP2C19 activity might be higher in men than in women [21].

Recently, gender differences in (S)-mephenytoin 4'-hydroxylase have been found in a Chinese population [7]. Surprisingly, in this Oriental population CYP2C19 activity was higher in female EMs as compared to male EMs. This is in contrast with our data and the data reviewed by Harris et al. [21].

In the study reported by Xie et al., the women included in the study were non-oral contraceptive users. In our design, however this was no exclusion criterion. It has already been suggested that for CYP2C19, an age-specific effect of gender might be based on the use of oral contraceptives [23]. Our data support this assumption since the mean S/R-ratio significantly increased with 68% in a group of OC users compared to non-OC users. Therefore, use of OCs is likely to decrease the clearance of typical CYP2C19 drugs such as e.g. omeprazole or diazepam or may decrease the efficacy of drugs like proguanil. Although OC use proved to be an important factor in explaining gender differences for CYP2C19 other factors have to be present. This is proved by the comparison between males and non-OC using females in which a significant reduced CYP2C19 activity was found in females.

In conclusion, our study shows polymorphism of CYP2D6 and CYP2C19 enzyme activity in a sample of the Dutch population that is in accordance with and extends previously published data. For CYP2D6 it was found that there is a large variation within a phenotyping class, indicating the existence of important co-factors in the metabolism of dextromethorphan. Gender differences exist for both CYP2D6 and CYP2C19. In contrast to CYP2C19, for CYP2D6 there is no apparent age dependency. For CYP2C19 gender differences among extensive metabolizers are apparent and the observed decrease in enzyme activity is associated with the use of oral contraceptives. Further studies have to be performed in this field of research.

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3.2 THE PREVALENCE OF CYP2D6 AND CYP2C19 GENOTYPES IN A POPULATION OF HEALTHY DUTCH VOLUNTEERS AND PSYCHIATRIC PATIENTS[#]

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3.2.1 Abstract

Aim: To estimate the prevalences of genetic polymorphism on CYP2D6 and CYP2C19 in a sample of the Dutch population and to compare this with the prevalences in other European regions. To assess the predictability of the genotyping strategy.

Methods: one thousand and twenty six (1026) unrelated subjects (765 healthy volunteers and 261 psychiatric patients) were evaluated for their CYP2D6 and CYP2C19 status. Volunteers were phenotyped using dextromethorphan (22 mg) and mephenytoin (100 mg). The urinary ratios of dextromethorphan over dextromethorphan were assessed by HPLC and the urinary (S)-mephenytoin over (R)-mephenytoin ratio (S/R-ratio) was assessed by gaschromatography. Genotyping was performed by PCR on the most common null alleles for CYP2D6 and CYP2C19 and on the gene duplication mutation for CYP2D6.

Results: for CYP2D6 the most frequently observed null allele was CYP2D6*4 which accounted for 89% of all null alleles. The prevalence of the gene duplication allele was 4.6%. The prevalence of poor metabolizers (PMs) in healthy volunteers was 5.5%, which was significantly lower as observed previously by phenotyping (8.0%; χ^2 test $p = 0.009$). For CYP2C19*2 and CYP2C19*3 a frequency of 13.3% and 0.2%, respectively, was observed. The S/R-ratio was significantly higher in heterozygous subjects (S/R-ratio = 0.22) compared to homozygous wild type subjects (S/R-ratio = 0.11). Comparison of all subjects below 45 years showed a significantly higher S/R-ratio in females compared to males, especially in heterozygous subjects (S/R-ratio: 0.39 vs. 0.19; $p < 0.001$). Above 45 years no significant differences existed between the two genders.

[#]: This paper has been submitted for publication to the European journal of Clinical Pharmacology

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Conclusions: the frequencies of CYP2D6 and CYP2C19 allelic variants were in accordance with other European populations. Assessment of *3, *4, *6, *7 and *8 alleles for *CYP2D6* and *2 and *3 for *CYP2C19* predicted the phenotype with an accuracy of over 98.6%. The data suggest a selective loss of CYP2D6 PM individuals from a volunteer pool for participation in clinical drug trials. Gene-dose effect existed for CYP2C19. CYP2C19 heterozygous females had a decreased CYP2C19 activity that is at least partly due to the use of oral contraceptives.

3.2.2 Introduction

Genetic polymorphism of drug metabolising enzymes (DMEs) often causes considerable pharmacokinetic variation between individuals leading to altered drug response and drug toxicity.¹ Cytochrome P450 isoenzymes CYP2A6, CYP2C9, CYP2C19 and CYP2D6 are polymorphically expressed due to variation in the genes coding for these enzymes². Cytochrome P450 2D6 (CYP2D6) catalyses the oxidative biotransformation of more than 40 clinically important drugs among them several CNS drugs and cardiovascular agents.² The in-vivo activity may vary between individuals from ultra rapid metabolism (UM), extensive metabolism (EM), intermediate metabolism (IM) to poor metabolism (PM). This variability is associated with about 50 mutations in the highly polymorphic *CYP2D6* gene locus³. The *CYP2D6* gene is located on chromosome 22 and is part of the CYP2D gene cluster with one functional gene (*CYP2D6*) and two pseudogenes (*CYP2D7P* and *CYP2D8P*). From several large studies in Caucasians (in total 1456 Europeans) it can be estimated that for *CYP2D6* 67% of all alleles encode for enzymes with normal activity, 4% encode for enzymes with decreased activity, 27% lead to non-functional enzymes and 2% encode for increased activity.^{3,4,5} The most common null alleles are *CYP2D6**4 (about 71% of null alleles), *CYP2D6**5 (about 16% of null alleles), *CYP2D6**3 (about 6% of null alleles) and *CYP2D6**6 (about 4% of null alleles) all the other null alleles are rare and have a prevalence of 1% or lower. Allelic variants that encode for enzymes with decreased activity are rare in Caucasians and the most common alleles are *CYP2D6**9 and *CYP2D6**10, both accounting for about 2% of all alleles. Ultra rapid metabolism (*CYP2D6**2x2) is caused by multiple functional *CYP2D6* genes causing an increased amount of CYP2D6 to be expressed. It was proposed that this gene duplication occurs as a reciprocal of gene deletion (*CYP2D6**5), which is thought to be caused by homologous, unequal recombination.⁶ Therefore, the prevalence of both gene duplication and gene deletion are likely to be similar.

A homozygous combination of null alleles leads to the PM phenotype, whereas a heterozygous wild type or combinations of alleles with decreased enzyme activity lead to reduced CYP2D6 activity. In-vivo enzyme activity can be assessed by measurement of the metabolic ratio of an enzyme-specific probe; for CYP2D6 dextromethorphan, sparteine, debrisoquine and metoprolol have been described as probe drugs^{7,8}. The prevalence of CYP2D6 PM phenotype differs per race and is reported to be 5 to 10% in white populations and 1 to 2% in Asians.²

Deficiency of cytochrome P450 2C19 (CYP2C19) occurs with a prevalence of PMs of 1-3% among white Europeans and about 20% among Orientals.⁹ Mephenytoin, an anti-convulsant drug, is a well-known probe drug for CYP2C19.¹⁰ The (S)-enantiomer of mephenytoin (MEP) is a substrate for CYP2C19 and the urinary ratio of (S)-MEP over (R)-MEP (S/R-ratio) after administration of racemic MEP can be used to identify PMs. Other probes for CYP2C19 are the proton pump inhibitor omeprazole¹¹ and the anti-malarial drug proguanil¹². Two null-alleles in the CYP2C19 gene have been described to account for approximately 83% of all PMs in Caucasians and 100% of all PMs in Orientals.^{13,14} In addition, three null alleles (CYP2C19*4, CYP2C19*5 and CYP2C19*6) have been described, but the frequencies of these alleles are expected to be below 1% in Caucasians.¹⁵

Recently, we reported the prevalence of PMs by phenotyping in a sample of the Dutch population (n = 4301) for CYP2D6 and CYP2C19 to be 8.0% and 1.8%, respectively.¹⁶ In the same study pronounced gender differences for CYP2C19 activity were observed and these differences could be associated with the use of oral contraceptives.

The aim of this study was to estimate the prevalence of genetic polymorphism on CYP2D6 and CYP2C19 in a sample of the Dutch population by genotyping and to compare this with the previous obtained phenotyping data and the prevalences in other European regions. In addition, the predictability of the genotyping strategy was assessed by comparing the actual phenotype with the predicted phenotype (genotype).

3.2.3 Methods

Subjects

One thousand and twenty six (1026) unrelated subjects (765 healthy volunteers and 261 psychiatric patients) were evaluated. Healthy volunteers were genotyped and phenotyped in the context of various clinical pharmacology studies and for the psychiatric patients genotyping only was performed as part of the pharmacotherapy. Patients were selected from a population of psychiatric inpatients of the psychiatric hospital Dennenoord (Zuidlaren, The Netherlands). A medical ethics committee approved the study. Samples (EDTA blood) for genotyping purposes were obtained by direct venapuncture. Healthy volunteers were phenotyped using mephenytoin (100 mg) in a cocktail with dextromethorphan (22 mg) and caffeine (100 mg) as described previously¹⁶. All urine was collected for 8 hours post-dose and a 20 mL aliquot was stored at -20 °C until analysis.

Bioanalysis

Urine samples were analysed as described previously.¹⁶ In short, dextromethorphan and dextrorphan were analysed by reversed phase high performance liquid chromatography. Sample preparation was performed by liquid-liquid extraction with acid hydrolysis. Quantitation was performed by fluorescence detection. Based on urinary dextromethorphan/dextrorphan ratios,

subjects with metabolic ratios ≥ 0.3 were classified as poor metaboliser (PM) and ratios < 0.3 were classified as extensive metabolisers (EM).¹⁷ (S)-mephenytoin and (R)-mephenytoin were analysed by an enantioselective capillary gas chromatography assay. Sample preparation was performed by liquid-liquid extraction with diethyl ether. Quantitation was performed using a nitrogen-phosphorus selective detector. The urinary (R)-mephenytoin and (S)-mephenytoin excretion ratio was determined and subjects with S/R-ratios ≥ 0.8 were classified as PM, whereas subjects with ratios < 0.8 were classified as EM.¹⁹ In addition, CYP2C19 PMs were reanalysed after acidification of the urine with HCl (12M) before extraction to confirm the PM status.²⁰

Genotyping procedures

Genotyping was performed on the *CYP2D6* gene and the *CYP2C19* gene using DNA isolated from whole blood samples. DNA was isolated using the QIAamp[®] DNA mini kit (Westburg BV, Leusden, The Netherlands). *CYP2D6* was investigated on the five most common allelic variants using a long-range polymerase chain reaction (PCR), amplifying the whole *CYP2D6* gene followed by a multiplex allele-specific PCR.²¹ The following allelic variants were investigated: *CYP2D6**3 (A), *CYP2D6**4 (B), *CYP2D6**6 (T), *CYP2D6**7 (E), *CYP2D6**8 (G). Unless stated otherwise, all reagents were obtained from Amersham Pharmacia Biotech. In short, the long amplicon of 4666 base pairs was amplified using 100 ng genomic DNA, primers "P1-5" and "n" at a concentration of 0.300 μ M each, 5 μ L of 10x Taq extender buffer (Stratagene), 400 μ M of each dNTP and each 2.5 units of Taq Polymerase and Taq Extender (Stratagene) in a final volume of 50 μ L. Amplification was performed in a Progene thermocycler (Technique Cambridge Ltd., Duxford, UK). Initial denaturation (94 °C, 2 min) was followed by 30 cycles of denaturation (94 °C, 0.5 min), annealing (62 °C, 0.5 min) and extension (72 °C, 4.5 min) followed by a final extension step (72 °C, 7 min). The pre-amplicon was 1:3 diluted with water and 2 μ L was used as template for two separate PCR reactions for the multiplex allele-specific PCR. The added primers were either wild type (A1[72 nM], B1[104 nM], E3[88 nM], T1[480 nM], G1[80 nM], M[800 nM]) or mutation-specific primers (A2[72 nM], B2[800 nM], E4[88 nM], T2[36 nM], G2[360 nM], M[800 nM]). Furthermore, the reaction mixture (50 μ L) contained 10x PCR buffer without MgCl₂, 1.5 mM MgCl₂, 250 μ M of each dNTP and 1.3 units Taq polymerase (Promega). The PCR mix was amplified as follows: Initial denaturation (94 °C, 2 min) was followed by 20 cycles of denaturation (94 °C, 2 min), annealing (55 °C, 0.5 min) and extension (72 °C, 3.3 min) followed by a final extension step (72 °C, 4.5 min). The products were analysed by a 2.5% agarose NA and Gelstar[®] nucleic acid staining (FMC Bioproducts). In addition, the presence of gene duplication that may lead to ultra rapid metabolism was analysed by an allele specific PCR⁶ and was performed as described by Steijns et al.²² Homozygous mutant genotypes were predicted to be the poor metaboliser phenotype. A subject is assigned as UM if gene duplication was detected and mutant alleles (as assessed with the long range PCR as described above) were

absent. In all other cases, the subject was assumed to have a normal CYP2D6 activity (EM).

The two most common allelic variants (*CYP2C19*2* and *CYP2C19*3*) of *CYP2C19* were investigated using a PCR with restriction analysis.^{13,14} In short, for *CYP2C19*2* the sequence of the forward primer was AATTACAACCAGAGCTTGGC and the reverse TATCACTTTCCATAAAAGCAAG and for *CYP2C19*3* the forward sequence was AAATTGTTTCCAATCATTTAGCT and reverse ACTTCAGGGCTTGGTCAATA. The amplicon of *CYP2C19*2* and *3 were prepared separately using the appropriate primer set at a concentration of 0250 µM each. Furthermore, the PCR mix contained 200 ng genomic DNA, 200 µM of each dNTP, 2x PCR buffer and 5 units of Taq Polymerase in a final volume of 50 µL. The PCR mix was amplified as follows: pre-heating (40 °C, 2 min), initial denaturation (94 °C, 5 min) was followed by 32 cycles of denaturation (94 °C, 1 min), annealing (54 °C, 1 min) and extension (72 °C, 1 min) followed by a final extension step (72 °C, 5 min). The products were restricted with SmaI (*CYP2C19*2*) and BamHI (*CYP2C19*3*) by overnight incubation at 25 °C and 37 °C, respectively. The products were analysed by a 3% agarose NA and Gelstar® nucleic acid staining (FMC Bioproducts). Homozygous mutant genotypes were predicted to be the poor metaboliser (PM) phenotype. In all other cases the subject was assumed to have a normal activity (EM).

Statistics

Data handling was performed in Microsoft excel® and all statistics were performed with SPSS® for windows (release version 9.00). Prevalences were compared with the chi-square test (χ^2) and metabolic ratios were compared by univariate ANOVA with post hoc analysis (LSD and Bonferroni tests) or by application of the Students t-test on genotype, gender and age.

3.2.4 Results

Demographics

The majority of the healthy volunteers were males (82%) of Caucasian origin (98%). The age differed from 18 to 79 years and the mean age was 33 years. The hospitalised psychiatric patients were not screened for racial origin. Patients were hospitalised for psychotic conditions (mainly schizophrenia and schizoaffective disorders), mood disorders, anxiety states, some forms of epilepsy and severe personality disorders. Of this population 45 % were males and the age varied between 18 and 92 years with an average age of 53 years.

CYP2D6 Genotype and Phenotype

The prevalence of non-functional allelic variants for *CYP2D6* was found to be 20.7 % in healthy volunteers and 24.7 in psychiatric patients (Table 1). The most frequently observed null allele was *CYP2D6*4* which accounted for 89% of all null alleles measured.

Table 1: Frequency of CYP2D6 and CYP2C19 null alleles from a sample of the Dutch population

<i>Gene</i>	Healthy Volunteers		Psychiatric Patients	
	n	% (95% CI)	n	% (95% CI)
<u><i>CYP2D6 Allele</i></u>	n = 765		n = 261	
<i>wt*</i>	1213	79.3 (77.3 – 81.3)	393	75.3 (71.6 – 79.0)
<i>*3</i>	28	1.8 (1.1 – 2.5)	14	2.7 (1.3 – 4.1)
<i>*4</i>	282	18.4 (16.5 – 20.3)	115	22.0 (18.5 – 25.6)
<i>*6</i>	6	0.4 (0.1 – 0.7)	0	0 (0 – 0.6)
<i>*7</i>	1	0.1 (0 – 0.3)	0	0 (0 – 0.6)
<i>*8</i>	0	0 (0 – 0.2)	0	0 (0 – 0.6)
Total mutant	317	20.7 (18.7 – 22.7)	129	24.7 (21.0 – 28.4)
<u><i>Gene Duplication</i></u>			n = 228	
<i>not present</i>	NA	-	208	95.4 (92.6– 98.2)
<i>wt*2n</i>	NA	-	6	2.8 (0.6 – 5.0)
<i>wt or *3*2n</i>	NA	-	1	0.5 (0 – 1.4)
<i>wt or *4*2n</i>	NA	-	3	1.4 (0 – 2.9)
Total Gene Duplication	NA	-	10	4.6 (1.8 – 7.4)
<u><i>CYP2C19 Allele</i></u>	n = 745		n = 238	
<i>wt*</i>	1289	86.5 (84.7 - 88.2)	411	86.3 (83.2 – 89.4)
<i>*2</i>	198	13.3 (11.6 -15.0)	65	13.7 (10.6 –16.8)
<i>*3</i>	3	0.20 (0 – 0.43)	0	0% (0 – 0.6%)
Total mutant	201	13.5 (11.8 – 15.2)	65	13.7 (10.6 – 16.8)

* wt = none of the null alleles tested were present; NA: not available

All the other null alleles were observed, except for CYP2D6*8, and the prevalence ranged from 8% (*3) to 0.3% (*7). In healthy volunteers the prevalence of homozygous mutant subjects (predicted poor metabolisers) was 5.5% (95% CI: 3.9 - 7.1%) and in psychiatric patients it was 7.7% (95% CI: 4.5 - 10.9%; Table 2). The prevalence of homozygous mutant subjects was higher in psychiatric patients but this was not significant (χ^2 test male: $p = 0.98$; female: $p = 0.13$).

Table 2: Prevalence of *CYP2D6* and *CYP2C19* genotypes in healthy volunteers and psychiatric patients in a sample of the Dutch population.

Genotype:		wt/wt	wt/mut	mut/mut	
Prevalence <i>CYP2D6</i> (%)					
Healthy Volunteer	Male	64.7	29.4	5.9	N = 626
	Female	61.2	35.3	3.6	N = 139
	Total	64.1	30.5	5.5	N = 765
Psychiatric Patient	Male	63.6	30.5	5.9	N = 118
	Female	53.8	37.1	9.1	N = 143
	Total	58.2	34.1	7.7	N = 261
Prevalence <i>CYP2C19</i> (%)					
Healthy Volunteer	Male	75.5	22.0	2.5	N = 605
	Female	74.0	22.9	3.1	N = 131
	Total	75.3	22.1	2.6	N = 736
Psychiatric Patient	Male	72.0	26.2	1.9	N = 107
	Female	77.9	19.1	3.1	N = 131
	Total	75.2	22.3	2.5	N = 238

wt/wt: homozygous wild type; wt/mut: heterozygous mutant; mut/mut: homozygous mutant

In addition, no significant differences existed between males and females (χ^2 test volunteers: $p = 0.27$; patients: $p = 0.26$). Compared to the prevalence of *CYP2D6* PMs estimated in an earlier study by phenotyping (8.0%)¹⁶, the prevalence of PMs in volunteers (5.5%) was significantly lower (χ^2 test $p = 0.009$). For psychiatric patients no such a difference was observed (χ^2 test $p = 0.49$). The large majority of the genotypes correctly predicted the phenotype as assessed by the dextromethorphan test (Figure 1). Five subjects (0.8%) had an EM genotype (wt/wt ($n = 1$) or wt/*4 ($n = 4$)), whereas the phenotype indicated PM (false negative) and three subjects (0.5%) had a PM genotype (*4/*4 ($n = 2$) or *4/*3 ($n = 1$)) but the phenotype indicated EM (false positive). The predictability of the test can therefore be estimated at 98.7%. Figure 2 shows that the mean metabolic ratio for dextromethorphan was clearly higher in homozygous mutant subjects (PMs) than in others ($P < 0.001$) but no significant differences were observed between homozygous and heterozygous wild types ($P = 0.33$).

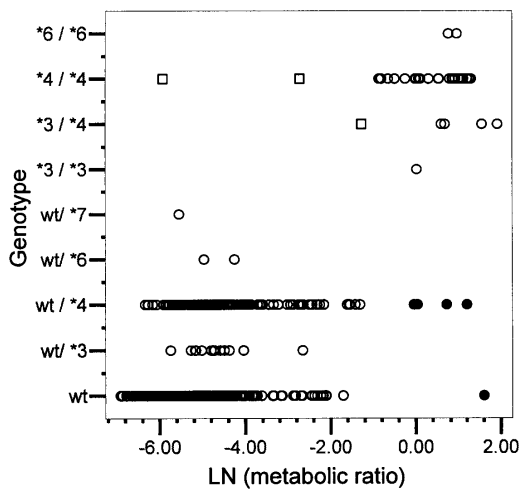


Figure 1: Distribution of dextromethorphan metabolic ratio among 634 Dutch healthy volunteers in the relation to their CYP2D6 genotype. wt indicates that none of the null alleles tested were present; open circles: concordance between genotype and phenotype; solid circles: false positive genotype. Antimode between extensive and poor metaboliser: LN (metabolic ratio) = -1.2.

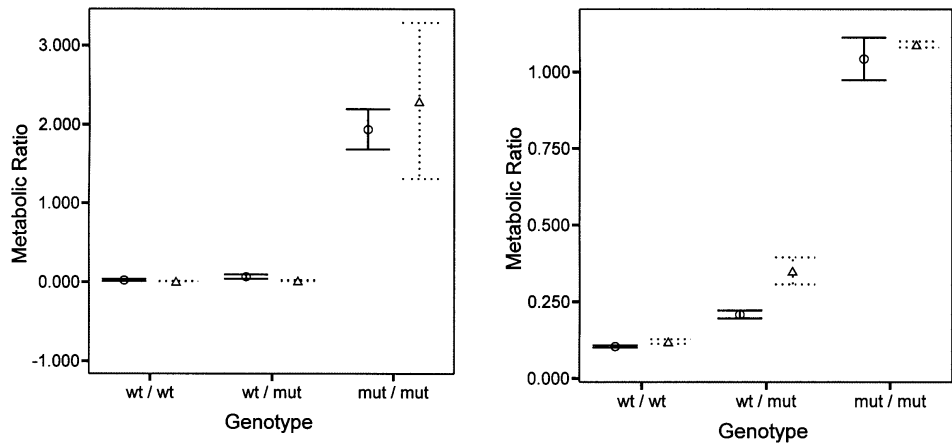


Figure 2: Mean metabolic ratios for CYP2D6 (Left Panel) and CYP2C19 (Right Panel) classified per genotype and per gender. Circles: males; triangles: females; error bars show mean ± standard error of mean; wt / wt: homozygous wild type; wt / mut: heterozygous wild type; mut / mut: homozygous mutant

The prevalence of the gene duplication allele was 4.6% in psychiatric patients (Table 1). In healthy volunteers the gene duplication was not assessed. The majority of the subjects carrying this allelic variant had a homozygous wild

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genotype (6 out of 10) and therefore the predicted prevalence of ultra rapid metabolisers is 2.8% (95% confidence interval: 0.6% - 5.0%). Of the remaining subjects having a gene duplication three subjects had a CYP2D6*wt / CYP2D6*4 genotype and one subject had a CYP2D6*wt / CYP2D6*3 genotype.

CYP2C19 Genotype and Phenotype

In healthy volunteers a prevalence of 13.3% and 0.2% was observed, for CYP2C19*2 and CYP2C19*3 respectively. In psychiatric patients the frequency for CYP2C19*2 was 13.7% whereas CYP2C19*3 was not observed. The prevalence of homozygous mutant subjects (PMs) did not differ between healthy volunteers (2.6 %) and patients (2.5%) (χ^2 test: $p = 0.58$). The majority of the genotypes correctly predicted the phenotype using mephenytoin (Figure 3). Two subjects (0.4%) had an EM genotype (wt/*2) whereas the phenotype indicated PM (false negative) and one subject (0.2%) had a PM genotype (*2/*2) while the phenotype indicated EM (false positive). The predictability of the test can therefore be estimated at 99.4%. The mean metabolic ratio for CYP2C19 was significantly higher (students t-test $p < 0.001$) in heterozygous mutant subjects (mean S/R-ratio = 0.22) compared to homozygous subjects (mean S/R-ratio = 0.11).

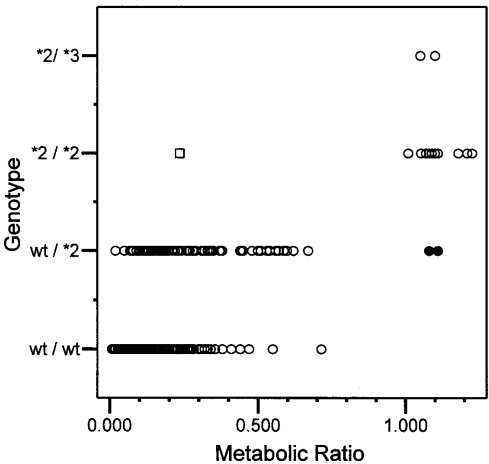


Figure 3: Distribution of mephenytoin S/R-ratio among 520 Dutch healthy volunteers in the relation to their CYP2C19 genotype. wt indicates that none of the null alleles tested were present; open circles: concordance between genotype and phenotype; solid circles: false negative genotype; square: false positive genotype. Antimode between extensive and poor metaboliser: metabolic ratio = 0.80.

CYP2C19 Gender Differences

The allelic frequencies of the most common CYP2C19 variants did not differ between males and females (χ^2 test volunteers: $p = 0.90$; patients: $p = 0.38$; Table 2). The mean S/R-ratio in EMs however, was significantly higher in females (S/R-ratio = 0.17) compared to males (S/R-ratio = 0.13; $p < 0.001$)

indicating a lower CYP2C19 activity in females (Figure 2). These differences did not exist for PMs and were most pronounced for the heterozygous mutant genotype for which the S/R-ratio was about 60% higher in females whereas in homozygous wild type subjects the S/R-ratio was only about 15% higher. Figure 4 shows the distribution of the metabolic ratio in relation to the age of subjects. It shows that differences between males and females for the homozygous wild type genotype were minor without a clear relation to the age of the subjects. In heterozygous subjects, however, a clear difference below 45 years was observed but this difference disappeared in older subjects. Comparison of all subjects below 45 years showed a significantly higher ratio in females compared to males for both homozygous wild type subjects (S/R-ratio: 0.12 vs. 0.10; $p = 0.013$) and heterozygous mutant subjects (S/R-ratio: 0.39 vs. 0.19; $p < 0.001$). In contrast, no significant differences existed between the groups above 45 years ($p > 0.10$).

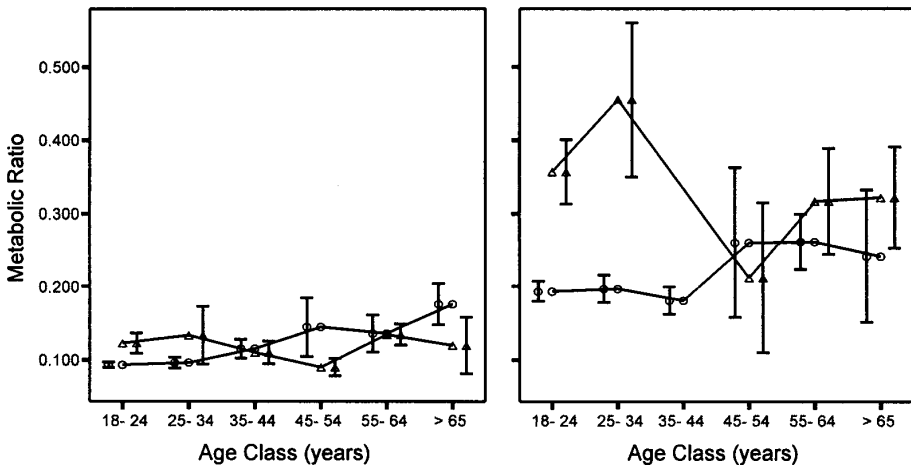


Figure 4: Mean metabolic ratios values for CYP2C19 per genotype: homozygous wild type (Left Panel) and heterozygous mutant (Right Panel) and classified per 10 year age groups. Circles: Males; Triangles: Females; Error bars show mean \pm standard error of mean.

3.2.5 Discussion

The prevalence of mutant alleles for CYP2D6 was found to be 21.7%. The gene deletion allele (CYP2D6*5) was not identified by our genotyping strategy. Absence of any amplification product in our assay indicated that this genotype might be involved but this should be confirmed by additional PCR tests^{4,23}. In our data set two subjects (0.2%) were found in which no amplification could be performed, although normal quality DNA could be isolated. Assuming that these subjects were homozygous CYP2D6*5, the allelic frequency can be estimated to be 5% of all alleles, which is comparable to other Caucasian populations.^{3,4,5}

However, being unable to identify gene deletion will not increase the risk of false negative EMs, because a combination with other null alleles will be characterised as homozygous mutant and will therefore be designated as PM. The frequency of *CYP2D6*4* (about 89% of all null alleles) was high compared to other European studies but this may be because the *CYP2D6*4 / CYP2D6*5* genotype will be detected as homozygous *CYP2D6*4*.

The prevalence of PMs in healthy volunteers observed in this study (5.5%) was low compared to psychiatric patients (7.7%), and low compared to the results from healthy Dutch volunteers by phenotyping (8.0%¹⁶ and 7.6%²⁴). The predictability of the genotyping assay (98.7%) makes it unlikely that this lower prevalence is caused by missed mutations, since this would have led to a larger number of false negatives. The number of non-Caucasians in our study population was too low (2%) to have significant influence. The prevalence PMs of the Caucasians in our population was 5.4% (95% CI: 3.6 – 7.2%). It has been suggested that databases of volunteers at drug study units do not reflect the general population due to a selective loss of PMs²⁵. This suggestion was based on a study in which a lower PM prevalence was observed in healthy volunteers (1.1%; n = 188) compared to the general population (6.3%; n = 142). It was hypothesised that when volunteers experience adverse events this might make participation in a subsequent trial less likely and therefore those who are more likely to experience adverse events (PMs) will drop out of the volunteer population. In our database the prevalence of PMs in a subpopulation of volunteers of 18 years (n = 37), the minimum age at which volunteers are allowed to participate in clinical trials, was 8.1% (95% CI: 0 - 17%). This seems to confirm the above-mentioned assumption but it should be noted that the observed difference with the total population could have occurred by chance (χ^2 test between 18 years and others: p = 0.33). In addition, if this bias is common for healthy volunteers in clinical drug trials, it cannot be explained why the prevalence of PMs in the phenotype database was 8.0%. Nevertheless, estimation of general population prevalences from healthy volunteer databases should be made with caution.

The prevalence of *CYP2D6* gene-duplication (4.6%) was in accordance with the expected incidence in Caucasians, since up to 7% may demonstrate gene-duplication⁶. Furthermore, the observed prevalence of *CYP2D6* genes carrying duplicate active genes (2.8%; 95% CI: 0.6 – 5%) was in good accordance with another study in Dutch psychiatric patients in which the prevalence was found to be 3.5%.²²

The prevalence of genetic polymorphisms of the *CYP2D6* gene did not differ between healthy volunteers and psychiatric patients. Several studies have been performed to associate the genotype with the risk of onset of various psychiatric diseases. Common psychiatric diseases like schizophrenia, Parkinson's disease, Alzheimer's disease did not show a clear association between *CYP2D6* polymorphism and the risk of onset of disease.²⁶ Therefore, no differences between healthy volunteers and psychiatric patients were expected and our observation is in line with this.

The frequency of the most common allelic variant *CYP2C19*2* (volunteers: 13.7%; patients: 13.3%) was comparable to other European populations: French 13%¹⁵, Portuguese 13%²⁷, German 13%²⁸ and Swedish 15%²⁹. The *CYP2C19*3* allele was observed twice in a subject of Oriental origin (*wt/CYP2C19*3* and *CYP2C19*2/CYP2C19*3*) and once in a Caucasian (*CYP2C19*2/CYP2C19*3*). The allelic frequency in Caucasians can therefore be estimated at 0.07% (95% CI: 0 – 0.2%) of all alleles and 0.5% (95% CI: 0.1- 0.9%) of all null alleles. In other European populations, the frequencies observed were somewhat higher and were found to be 0.2 to 0.3% of all *CYP2C19* alleles^{15,29}. In two heterozygous *CYP2C19*2/wt* samples false negative results were observed, which were most likely caused by other non-coding mutations. Hence, the frequency of other non-coding mutations can be estimated at 0.2% of all *CYP2C19* alleles in the Dutch population. The significantly higher S/R-ratio in heterozygous subjects compared to homozygous wild type subjects is most likely caused by the gene-dose effect of *CYP2C19*, which has been detected by others as well.^{28,30,31}

For routine genotyping to predict *CYP2C19* phenotype in Dutch Caucasians assessment of *CYP2C19*2* seems to be appropriate. The risk of false negatives is about 0.3% for a heterozygous subject and the risk on false negatives can be neglected for homozygous wild type subjects (1 out of ten thousand results). When subjects are of Oriental origin, additional testing for *CYP2C19*3* is necessary to rule out a false negative result.

The S/R-ratios in females were clearly higher than in males, especially for heterozygous subjects, indicating a lower *CYP2C19* activity for females compared to males. This confirms our earlier observations and those of others.^{16,30,31} This study shows that these gender differences could not be accounted for by genotypes because the frequency of mutant alleles was the same for males and females in both healthy volunteers ($p = 0.90$) and in psychiatric patients ($p = 0.38$). In our previous study we have shown that the use of oral contraceptives (OC) is an important factor in these gender differences. For proguanil, another *CYP2C19* substrate, it has been proven that its metabolic ratios were higher in females taking exogenous hormones (OCs or hormone replacement therapy) indicating a decreased *CYP2C19* activity.³⁰ Possible inhibition of *CYP2C19* by estrogens (both endogenous and exogenous) has been detected in several studies, e.g. inhibition of metabolism of diazepam or imipramine (both known *CYP2C19* substrates), and *in vitro* inhibition of s-mephenytoin 4'-hydroxylation by ethinylloestradiol and oestradiol.^{30,32,33,34} The present study supports these findings. Although no data on the use oral contraceptives were collected, the gender differences disappeared in the group above 45 years, for which OC use is less likely. Interestingly, gender differences were most pronounced for the heterozygous mutant genotype. This supports the hypothesis that *CYP2C19* is inhibited. If an individual is heterozygous the amount of *CYP2C19* expressed is decreased (gene-dose effect) and the individual is therefore more prone to inhibitory compounds than when the normal amount of enzyme is expressed (homozygous wild type). This observation may

be of clinical relevance because effects of CYP2C19 inhibition seem to be relevant in heterozygous subjects only, accounting for 13-14% of the total population.

In conclusion, the frequency of CYP2D6 and CYP2C19 allelic variants were in accordance with other European populations. Assessment of *3, *4, *6, *7 and *8 alleles for *CYP2D6* and *2 and *3 for *CYP2C19* predicted the phenotype with an accuracy of over 98.6%. Furthermore, the data suggested a selective loss of CYP2D6 PM volunteers recruited for clinical drug trials. No indications for an association between genetic polymorphism (CYP2D6 and CYP2C19) and psychiatric disease were observed. Gene-dose effect existed for CYP2C19. CYP2C19 heterozygous females had a decreased CYP2C19 activity, at least partly due to the use of OCs.

3.2.6 References

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